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THE TETRANUCLEATE EMBRYO SAC OF CLINTONIA

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(WITH PLATE V)

The following results are published in the belief that from the standpoint either of morphology or of phylogeny it is important we should become acquainted with the variations of the angiosperm embryo sac. By searching out and comparing all deviations from the normal type, we may hope to ascertain the directions in which the embryo sac is varying at the present time, and perhaps we may also discover some clue to the path along which it has come. The results here given were obtained from a study of *Clintonia borealis*, collected in the neighborhood of Toronto and of Lake Joseph, Ontario.

The youngest ovaries, collected May 9, showed the ovules already completely anatropous, each with a large archesporial cell, having its nucleus in the synapsis stage (fig. 1). The archesporial cell undergoes no cell division, neither cutting off a parietal cell nor dividing into megaspores, but, as in many liliaceous ovules, passes directely into the embryo sac. Its nucleus, however, suffers a twofold reducing division which is of considerable interest.

The nucleus in the synapsis stage is very large; it is usually situated slightly below the middle of the cell, and occupies fully four-fifths of its width. In the condition represented in fig. 2, the protoplasm is becoming denser about the periphery of the nucleus in preparation for spindle formation, and the loops of chromatic material are beginning to separate and spread throughout the nuclear space. Subsequently, when the fibrils of the spindle are quite distinct, the chromatin is found segmented into chromosomes, which frequently appear in the x's, y's, and other forms, characteristic of the heterotypic division.

The number of these chromosome pairs I have not been able to determine with certainty, since the nucleus is too large to be included in one section. Further, since all my sections are cut longitudinally to the ovule and therefore at right angles to the equatorial plate, it is difficult to make an accurate count of the chromosomes at a later stage of the mitosis. It is certain, however, that we are here dealing with the haploid number, probably 12. In vegetative divisions 20 or more chromosomes can easily be counted, though in this case also it is not easy to determine the number with certainty. The chromosomes when drawn into the equatorial plate are short and thick, almost globular, strongly contrasting in this respect with the long, crowded chromosomes of the vegetative division.

In none of my material, though careful examination was made, could there be found any difference in the separating chromosomes (fig. 3). Those going to the lower pole are fully as large and give the same staining reaction as those going to the upper pole. But on arriving at the poles of the spindle, the two groups behave very differently. Those at the upper pole unite into a normal nucleus, while those reaching the lower pole fuse together into an irregular lump, without spongioplasm or distinct nuclear membrane. Frequently chromosomes or chromosome fragments fail to reach the principal mass, and remain scattered along the spindle or in the cytoplasm outside. When Flemming's triple stain is used, these fragments, as well as the large chromatic lump, take only the safranin and appear semitransparent, while the chromatin of the upper nucleus, taking the gentian violet, appears dark and opaque. No wall is run in at the close of this division, but a distinct cell plate is formed by the thickening up of the spindle fibers (fig. 4).

Division follows in each of the daughter nuclei resulting from the heterotypic mitosis. My material, however, does not furnish any examples of the prophase of this division, nor any information with respect to the chromosomes. The telophase is represented in figs. 5 and 6. In both figures the remains of the first spindle and its cell plate are still distinguishable (on the left side of fig. 6). A cell plate is formed also on the second spindles, but these and the earlier cell plate are transient structures and disappear shortly.

The division of the upper nucleus results in this case also in the formation of an upper healthy nucleus and a lower irregular lump of chromatin. By the division of the lower of the daughter nuclei two such lumps are formed. Thus the embryo sac now contains an upper healthy nucleus and three lumps of chromatic material without spongioplasm. The commonest arrangement is that of figs. 6 and 7 rather than fig. 5, the healthy nucleus being somewhat above the middle of the cell and much smaller than the nucleus of the mother cell.

These successive divisions, especially the second, must be completed very rapidly, if one may judge from the rarity of their occurrence in the material. Thus, of about 350 ovules in the stages represented by figs. 2–7, more than 225 were in synapsis, and only 5 in the second division.

It can scarcely be doubted that the four nuclei just described are the four megaspore nuclei, and that megaspore formation in *Clintonia* differs from the normal type simply by omitting the formation of walls and by an earlier beginning of the degeneration of the sterile megaspores.

It is clear that division of the imperfect nucleus formed by the heterotypic mitosis can be of no importance in the subsequent history of the embryo sac; yet among 80 embryo sacs of the age of fig. 7, there was not one in which the lower nucleus had failed to divide. The three sterile nuclei could be found in every case. This fact would imply a strong hereditary tendency to a second division, such as we might expect to accompany megaspore formation; and incidentally it would indicate that the impulse to nuclear division must originate in the cytoplasm, since so imperfect a nucleus cannot be regarded as capable of exercising any of the functions of a normal nucleus.

A second peculiarity of the embryo sac of *Clintonia* is its unipolarity. Only two divisions of the megaspore nucleus occur, and thus are produced four nuclei which, in their position, relation to one another, and later behavior, exhibit the characteristics of the four upper nuclei of a normal embryo sac. The change from megaspore to mature embryo sac, involving two nuclear divisions, requires an interval of about three weeks, and it is therefore difficult to obtain karyokinetic figures. That those I have been able to secure are in the late phases is probably due to slow infiltration of the fixing medium.

The fertile megaspore nucleus moves to a higher position and rests for some time. The spindle of the first division is parallel to the axis of the embryo sac, and the two nuclei formed are always one above the other, as in figs. 8 and 9. Again at this division a temporary cell plate is formed. The second division occurs simultaneously in the two nuclei, and the spindles are at right angles to each other. The two upper sister nuclei and the protoplasm about them become the synergids; of the other two, one surrounded by vacuolated protoplasm and a plasma membrane becomes the egg, and the remaining one is a free nucleus, in position and appearance the upper polar.

Usually at these stages some remains of the sterile nuclei are still recognizable, but it is not always possible to be sure all three are present. They stain much less deeply than when first formed, taking little safranin and appearing to have a dark color of their own, independent of the stain. They vary considerably in size, and very frequently appear pitted or vacuolated, as in fig. 9. They are usually situated in the lower end of the embryo sac, as in figs. 8 and 11; fig. 9 is an exceptional case, since one of the sterile nuclei appears in the micropylar region.

Up to the tetrad stage the protoplasm of the embryo sac shows no tendency to unipolarity; it is coarsely granular and evenly distributed. But after the first division of the megaspore nucleus, when there is considerable enlargement of the sac, the protoplasm of the antipodal region becomes scant and stringy with large irregular vacuoles; that of the micropylar region is much denser, and the numerous vacuoles, which appear only at a late period, are small and globular.

A third peculiarity of *Clintonia* is its comparative sterility. Though it blossoms freely, only a very small proportion of the flowers result in fruit. Propagation by vegetative outgrowths of the rhizome is the common means of multiplication. An examination of 50 ovaries, collected one week after the opening of the flowers, disclosed no embryos and no certain proof that fertilization had occurred. In several embryo sacs one of the synergids was partially disintegrated, and in two cases two free nuclei were found below the egg apparatus, presumably derived from division of the polar nucleus. Whether or not fertilization occurs normally in those ovaries which develop into fruit, I am at present unable to say, nor can I assign the cause of the large proportion of abortive flowers. Apparently it is not due to any imperfection in the microspore, which contains two normal nuclei and appears plump and healthy.

An attempt to estimate the percentage of fertile flowers by field observation proved futile. An area producing 550 flowers was kept under observation and undisturbed, but all to no purpose. The flowers one and all were abortive, and two weeks after opening had withered and fallen away, leaving only the shriveled pedicels.

An attempt also was made to determine whether the sterility is due to imperfect pollination. A small number of flowers were artificially pollinated, but these like the others yielded no seeds. However, as several days of heavy rain interfered with the experiment, I cannot regard it as conclusive.

Discussion

The interpretation of the embryo sac of *Clintonia* is made easier by recent investigations of certain Onagraceae. GEERTS (**6**) finds that in *Oenothera Lamarckiana* the single archesporial cell gives rise to a tetrad row of megaspores, of which the uppermost develops, slowly absorbing the three lowermost and producing four nuclei arranged much as in *Clintonia*. The same condition is reported by MODILEWSKI (**10**) as occurring in *Epilobium angustifolium*, *E. Dodonaei*, *Oenothera biennis*, and *Circaea lutetiana;* in all these the unipolarity of the embryo sac is strongly marked and there is "double fertilization." A comparison of the embryo sac development of these and of *Clintonia* makes it clear that the four nuclei of figs. 5–7 represent four megaspores. Further, in these six species the unusual condition prevails of having the upper megaspore fertile. The chief differences shown in the development of *Clintonia* are in the absence of walls separating the megaspores and in the large proportion of sterile ovules.

A nearly similar embryo sac occurs in *Oenone* and *Mourera*, two genera of the Podostemaceae examined by WENT (**14**). A mother cell after synapsis divides into two; the upper of these after division of its nucleus gradually disintegrates; the lower cell also gives rise to two nuclei, one of which, the lower, becomes a mere clump of chromatin, while the other divides twice and the four resulting nuclei arrange themselves as in *Clintonia* and the above named Onagraceae. Though WENT does not discuss the theoretical value of the first four nuclei derived from the mother cell, it seems clear they represent megaspores, of which that next the innermost is the fertile one. Thus two megaspores appear in the upper cell, and two in the lower cell which becomes the embryo sac. The development of one of the middle megaspores, although uncommon, is not unknown. It has been seen in *Acacia* and *Eriobotrya* (GUIGNARD 1881, 1882), *Trapella* (OLIVER 1888), some of the Araliaceae (DUCAMP 1902), and *Asclepias* (FRYE 1902); to this list, which is taken from COULTER and CHAMBERLAIN'S *Angiosperms*, may be added *Vaillantia* and *Collipeltis* (LLOYD 1902).

In the examples thus far reviewed, the four functional nuclei of the embryo sac are the direct derivatives of one of four megaspores. Some cases of a different nature remain for consideration. In *Limnocharis* (HALL 1902) tetrads are not formed; the first two nuclei of the mother cell place themselves at opposite poles, and while the upper gives rise to the egg apparatus and a polar nucleus, the lower remains undivided. Nearly similar is *Helosis*, but in this case the primary antipodal nucleus soon degenerates (CHODAT and BERNARD 1900; I have not been able to consult the original paper). *Cypripedium* (PACE **11**) furnishes another example of a tetranucleate embryo sac. In this plant the mother cell divides once and the lower of the two daughter cells becomes the embryo sac, its nucleus undergoing two divisions. Miss PACE interprets the first two nuclei of the embryo sac as megaspore nuclei. Thus according to this view the embryo sac of *Cypripedium* is compound, being the product of two megaspore nuclei.

Miss PACE extended the conception of a compound embryo sac to *Lilium*, in which four megaspores are thought to function, and more recently COULTER (4) has extended it to all those cases in which tetrad formation is apparently suppressed, and especially to the 16-nucleate embryo sac of *Peperomia* and the like.

McAllister's (**7**) discovery of temporary walls separating the first four nuclei of the embryo sac of *Smilacina*, which otherwise resembles *Lilium*, is strongly confirmatory of this interpretation. Though the results of the present paper have no direct bearing upon this question, it may be pointed out that in *Clintonia* and the Onagraceae we see for the first time a mature gametophyte of four nuclei proceeding from an indubitable megaspore; and the occurrence in the Penaeaceae (Stephens **12**) and *Euphorbia procera* (Modilewski **9**) of four symmetrically placed groups of nuclei, each group similar in appearance to the gametophyte of *Clintonia*, certainly suggests a similar origin for each group. No case is yet known of a 16-nucleate embryo sac derived from one of four megaspores. In *Peperomia* (Brown **1**) and the Penaeaceae (**12**) there are no tetrads, and reduction occurs in the embryo sac. In *Euphorbia procera* the history has not been traced back to the mother cell. In Gunnera[1] also (Modilewski **8**, Ernst **5**) there are no tetrads. The case of *Pandanus* (Campbell **3**) offers some difficulty; the embryo sac is said to be one of three sporogenous cells (presumably megaspores). But Campbell did not obtain evidence where the reduction divisions occur, and the view that in the group of three "sporogenous cells" the upper two are parietal cells rather than megaspores is a fair inference from his figures and data. The point certainly needs further investigation.

Clintonia, *Eichhornia* (Smith 1898), *Avena* (Cannon 1900), and *Asperula* (Lloyd 1902) give us examples of four megaspores in one sac, and in *Crucianella* Lloyd (1902) found all four megaspores germinating within the one wall. No one doubts that these are megaspores, simply because three of them or their products disintegrate. But surely the weightier evidence is that of chromosome reduction, and this applies equally to *Lilium*, *Peperomia*, etc. This is the position taken by Coulter (**4**). He maintains that in the genesis of the angiosperm embryo sac "the essential part of the process is found in the first two divisions," and he adds "megaspores, at least their nuclei, cannot be omitted." Brown (**2**) thinks we cannot make chromosome reduction the sole test of megaspore formation, and he proposes a different criterion. "A distinction," he says, "between the first division of a megaspore and a division giving rise to megaspores is that while in the first case no cell plate is formed on the spindle, in the latter case either a wall or a cell plate is formed on the spindle." BROWN admits the compound nature of the embryo sac of *Lilium* and *Peperomia*, but for the reason just quoted refuses to admit it in the case of *Cypripedium.* To be consistent, he ought not to allow it for *Lilium*, since in this case a cell plate is formed in the third mitosis of the embryo sac. His distinction breaks down, however, in the case of *Clintonia;* in fig. 8 the first division of the megaspore nucleus is accompanied by a cell plate. It seems to the writer that the general principle of a compound embryo sac, while not altogether free from difficulties, furnishes the explanation of a large number of abnormal embryo sacs.

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[1] Ernst understands Schnegg (1902) to assert the occurrence of tetrads in *Gunnera Hamiltoni*, but the latter author does not figure tetrads nor use the word, and it seems probable his "Viertheilung" refers to nuclear and not to cell division.

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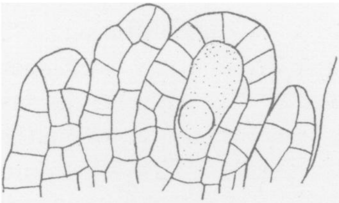

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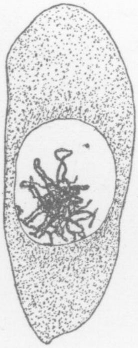

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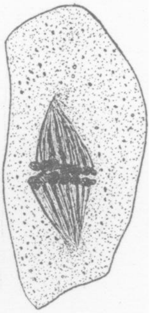

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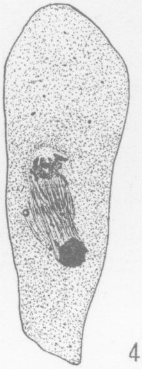

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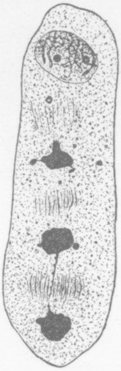

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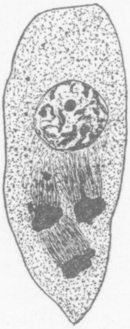

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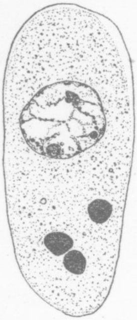

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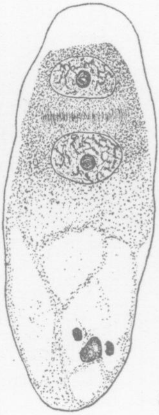

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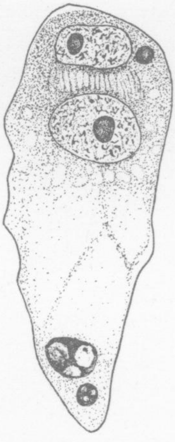

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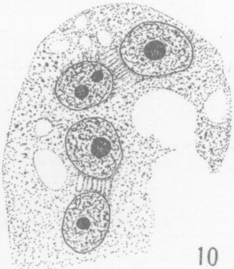

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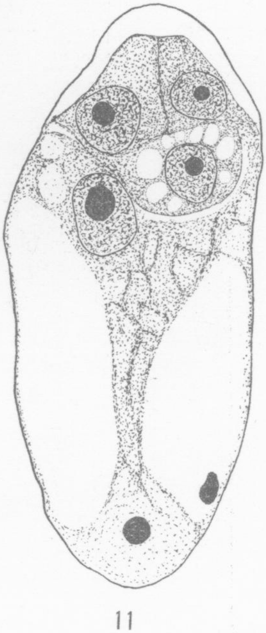

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EXPLANATION OF PLATE V

All the figures were drawn with the aid of an Abbé camera lucida on a Leitz microscope. For fig. 1, ocular 1 and objective 7 were used; for all the others, a 2 mm., 1.30 aper. apochromatic immersion lens with compensation ocular 4. The drawings have been reduced one-half in reproduction.

FIG. 1.—Apex of nucellus with megaspore mother cell.

FIG. 2.—Megaspore mother cell with nucleus emerging from synapsis.

FIGS. 3, 4.—First mitosis in megaspore mother cell.

FIGS. 5, 6.—Second mitosis in megaspore mother cell.

FIG. 7.—Megaspore mother cell with four megaspore nuclei, one healthy and three degenerate.

FIGS. 8, 9.—Embryo sac showing first division of fertile megaspore nucleus.

FIG. 10.—The apex of an older embryo sac.

FIG. 11.—Embryo sac of a fully opened flower; in this, as in all the others, there are no antipodals.